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Commentary

Vasculogenic Mimicry: How Convincing, How Novel, and How Significant?

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In a recent publication, Maniotis et al[1] report that blood vessels of malignant eye tumors known as uveal melanomas are formed by tumor cells instead of endothelial cells. The authors use the term vasculogenic mimicry to describe this phenomenon and consider it a novel concept in the biology of tumor vascularization. The paper has received widespread attention and apparent validation through two commentaries, one published along with the paper in *The American Journal of Pathology*[2] and another published concurrently in *Science.*[3]

Despite the paper's impact the evidence is, in our view, unconvincing. The problems are, however, not easily detected by readers unfamiliar with the background or pitfalls of this specialized topic. Although it is intriguing and worthy of further study, the evidence presented in Maniotis et al for a functionally significant contribution of tumor cell-lined blood vessels to vascularization and blood flow in uveal melanomas is neither persuasive nor novel. The purpose of this commentary is to examine the evidence for vasculogenic mimicry and the reasons for our assessment. (*Note:* This commentary does not address the *in vitro* or microarray data presented by Maniotis et al, because the interpretation of these results is dependent on the histological, immunohistochemical, and electron microscopic evidence that is the focus of our remarks. Also, this commentary does not question the validity of the relationship between the periodic acid-Schiff (PAS) staining pattern of uveal melanomas and clinical outcome, as reported by Folberg et al in several publications.[4,5] This correlation may be clinically useful even if the PAS staining pattern does not faithfully represent the microvascular architecture. Neither does our commentary question the usefulness of microvascular density as a prognostic factor for survival in uveal melanomas.[6,7] Indeed, PAS staining pattern and microvascular density may offer complementary indices of the lethality of these tumors.[6–9])

How Convincing?

A definitive demonstration of tumor cell-lined blood vessels would address several key questions. 1) Are the structures under consideration actually blood vessels, defined as routes through which blood circulates; ie, do they contribute meaningfully to blood flow? 2) Can the presence or absence of endothelial cells and tumor cells in contact with the vascular lumen be established using unambiguous markers? 3) If erythrocytes are used as markers, are they located inside or outside blood vessels? 4) Where is the interface between endothelial cells and tumor cells in blood vessel walls? 5) How extensive is the presumptive contribution of tumor cells to the lining of blood vessels?

The first two of these questions are addressed in Maniotis et al, but the approach is not on target and the results are not straightforward or convincing. Consider the following five problems.

PAS-Stained Networks in Uveal Melanomas Do Not Reflect the Microvascular Architecture

In a search for tumor cell-lined blood vessels, a key step is the identification of the vessels in question as routes for circulating blood. Maniotis et al used periodic acid-Schiff to stain the "patterned vascular channels" in histological sections of uveal melanomas. The interpretation of PAS-stained networks in uveal melanomas as reflecting the microvascular architecture of the tumors stems from a report in 1992 by Folberg et al[4] that defines nine different vascular patterns in these tumors based on PAS staining of what was assumed to be periendothelial basement membrane. The term network was used for the most complex pattern consisting of three or more PAS-stained

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back-to-back loops. Folberg, who is one of the authors of the Maniotis et al paper, applied this approach in numerous subsequent papers.[5,10–23] The presence of one or more PAS-stained networks was reported to indicate an unfavorable clinical outcome.[4,5]

This approach is not in concert with multiple lines of evidence showing that PAS-stained networks do not represent blood vessels, do not pinpoint the location of blood vessels, and do not define the microvascular architecture of uveal melanomas. Immunohistochemistry for the endothelial cell marker Factor VIII-related antigen shows scattered discrete profiles of blood vessels instead of loops and networks around clusters of tumor cells.[6,8] In response to this immunohistochemical evidence, Folberg[24] has argued that PAS staining, though not specific for blood vessels, precisely matches the microvascular pattern by staining the perivascular connective tissue. This argument is inconsistent with other evidence. For example, "silent" regions with no PAS staining, which Folberg et al [4] interpreted as avascular regions of the tumors, contain abundant vessels that are immunoreactive for Factor VIII[8] and another endothelial cell marker, CD34.[7] Furthermore, the pattern of CD34 immunoreactivity matches the pattern of Factor VIII staining regardless of whether PAS staining is present.[7,9] The apparent similarity of PAS staining to the staining pattern of the endothelial cell marker *Ulex europeus* agglutinin I lectin, as reported by Folberg,[4] is likely to be an artifact of connective tissue autofluorescence.[8]

Maniotis et al report that the PAS-stained pattern in tissue sections precisely matches the microvascular architecture seen in angiograms. However, geometric considerations dictate that vascular patterns seen in angiograms, which are 2-dimensional projections of 3-dimensional networks, should not match the pattern of blood vessels visible in corresponding thin histological sections, which are essentially 2-dimensional.[8] The structure of the PAS-stained networks as viewed in 2-dimensional sections indicates that they are curved sheets, not tubes or sinusoids (Figure 1). A 3-dimensional anastomosing network of tubes or sinusoids would appear in 2 dimensions as discontinuous segments of tubes, which when cut in various planes of section would range in appearance from circles or ellipses to longitudinal sections of cylinders (Figure 1). A network of blood vessels would not appear in 2 dimensions as a continuous, interconnected network of lines. Also, it is extremely unlikely that an angiogram would have precisely the same appearance as a histological section because the network of blood vessels shown in the angiogram should appear as discrete vessel profiles in thin histological sections (Figure 1). The same argument applies to ultrasound images of the tumors.[14]

Thus, the PAS-stained networks called "patterned vascular channels" are unlikely to represent networks of blood vessels. Instead, these networks appear to consist of septa of connective tissue and extracellular matrix around clusters of tumor cells. Vessels are located in some of the septa, but most of the continuous, interconnected dark lines around tumor cells shown in PAS-stained histological sections are not blood vessels.

If the PAS-stained pattern does not closely represent the arrangement of blood vessels, then indeed it would not be expected to match the distribution of endothelial cell markers. Therefore, the mismatch between the PAS pattern and endothelial cell distribution would be the expected finding rather than novel evidence of endothelial cell absence and blood vessel formation by tumor cells.

Endothelial Cell-Lined Blood Vessels Are Present in Uveal Melanomas

Maniotis et al conclude that blood vessels in aggressive uveal melanomas are formed by cancer cells, based in part on their failure to find any endothelial cells in these tumors. In their hands, immunoreactivity for the endothelial cell markers Factor VIII-related antigen, CD31, CD34, and KDR (flk-1) was weak, focal, and discontinuous, and most of the PAS-stained networks were unlabeled by these markers. CD34 immunoreactivity and KDR immunoreactivity were found in the lumen of blood vessels, not in the wall, and CD31 immunoreactivity appeared to be located in the nuclei of perivascular tumor cells.

In sharp contrast to these findings, several studies have documented the presence of Factor VIII-related antigen, CD31, and CD34 immunoreactivity of blood vessels in aggressive uveal melanomas.[6–9,16,18] Some of the authors of the Maniotis et al paper contributed to this evidence.[16,18] All of these papers show distributions of immunoreactivity that would be expected for tumor microvasculature, not PAS-stained networks. Because the PAS-stained septa are themselves not blood vessels, they would not be expected to have immunoreactivity for endothelial cell markers. Only the blood vessels within them and elsewhere would have these features. Although Maniotis et al acknowledge the presence of Factor VIII-related antigen, CD31, CD34, and KDR, they argue that these molecules are expressed by tumor cells, not endothelial cells. Yet, to fit the distribution of immunoreactivity, they argue that the expression is restricted to those tumor cells that line blood vessels. In our view, this argument is circular.

Immunoreactivity for Factor VIII-related antigen, CD31, CD34, and KDR and *Ulex* lectin histochemistry would be expected to show the location of vascular endothelial cells. Indeed, the focal, discontinuous regions of immunoreactivity illustrated by Maniotis et al fit the expected distribution of endothelial cells in tumor vessels. Surprisingly, however, this finding is interpreted as showing that the immunoreactivity was associated with the contents of the tumor vessels, not the vessels themselves. A more straightforward interpretation would be that the antibodies identified endothelial cells in tumor vessels that were collapsed or poorly preserved. Fixation by immersion in formalin combined with the high tissue pressure in tumors would predispose to vessel collapse.[25] Also, because the 234 tumors described in this study were removed before 1993 and the tumors were fixed in formalin and embedded in paraffin,[5] the preservation of the specimens was unlikely to be optimal for immunohistochemis-

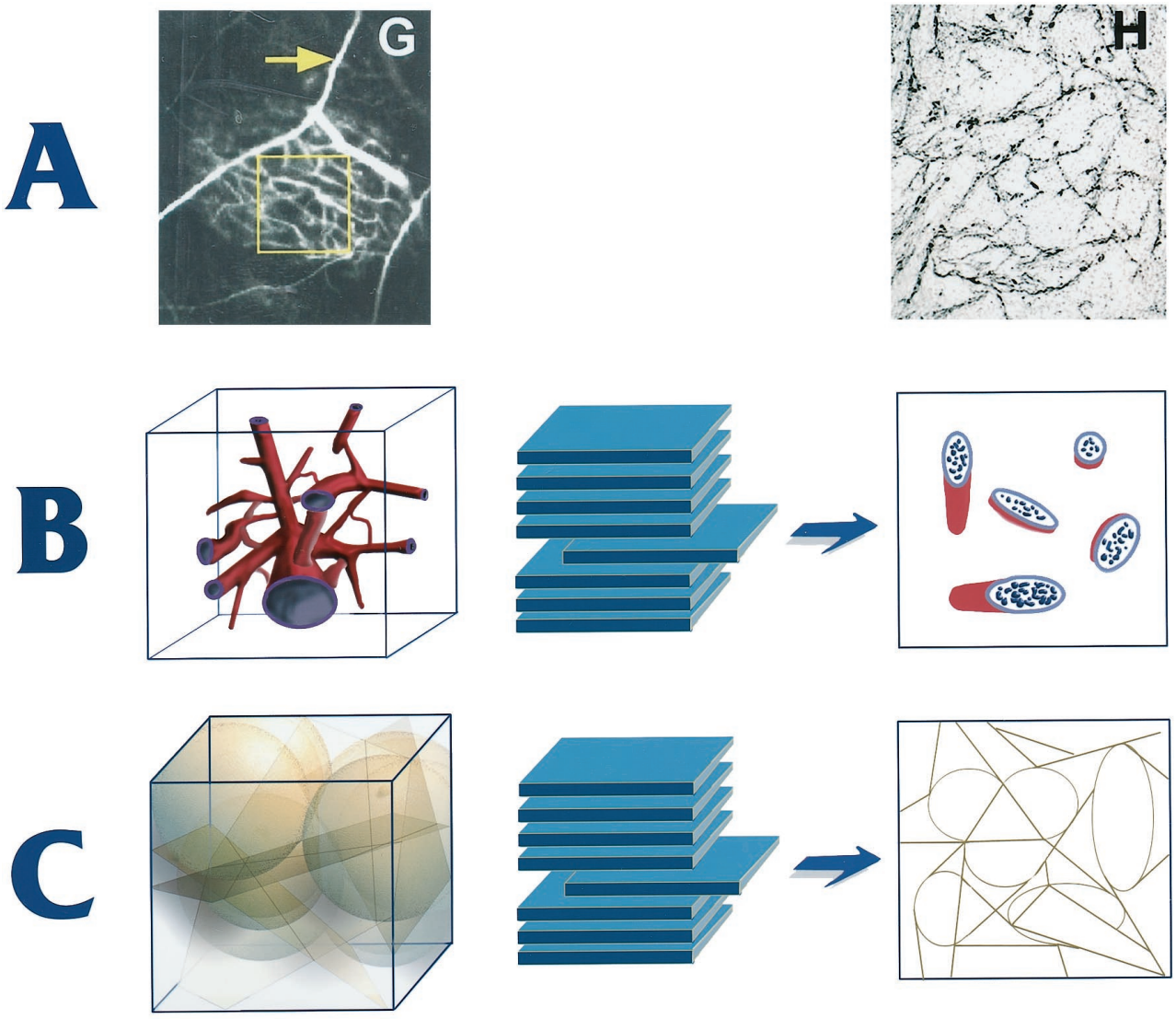

Figure 1. Relationship between 3-dimensional structures and individual 2-dimensional sections from the same structures. Figures in **A** are adapted from Maniotis et al. The left-hand figure, showing an angiogram of a uveal melanoma, is an image of a 3-dimensional structure and must correspond to the example in **B,** which illustrates a 3-dimensional network of interconnecting tubes (**left**) and the resulting ellipses seen in a section (**right**). The right-hand figure of **A**, however, is a 5-μm section of the tumor shown in the angiogram and resembles more closely the structure in **C**, which shows a hypothetical clustering of planar and ellipsoidal objects (**left**) and the interconnected networks seen in a corresponding section (**right**).

try at sufficient resolution. Bleaching of melanin to improve visibility in the histological sections would further degrade immunoreactivity.[8]

Extravasated Erythrocytes in Extracellular Matrix Can Be Misinterpreted as Vascular Channels

A convincing argument for the presence of blood vessels lined by tumor cells depends on the unequivocal identification of the structures in question as vessels connected to the bloodstream. In the Maniotis et al paper, key evidence for this identification came from transmission electron microscopic studies that produced the illustrated example of an alleged tumor cell-lined blood vessel. In this illustration, the "vessel" was identified by the presence of erythrocytes, and the "lumen" is lined by basement membrane. The possibility of extravascular erythrocytes was not considered.

There are three problems with this part of the argument. First, two earlier reports by Folberg and colleagues, both of which are cited in Maniotis et al, document the presence of endothelial cells in highly invasive uveal melanomas examined by transmission electron microscopy.[4,10] A recent report (Foss AJE, Munro P, Cree I, submitted for publication) confirms these earlier observations. Electron micrographs in the earlier papers by Folberg et al[4,10] clearly show endothelial cells that are in contact with the vessel lumen and are surrounded by basement membrane. The point is made that the endothelial cells do not have intact intercellular junctions and, therefore, do not have a normal barrier function.[4,10] By contrast, after examining the same cases, Maniotis et al

now claim that endothelial cells are not present at all and that the blood is in direct contact with basement membrane and tumor cells.

Second, the ultrastructural example in Maniotis et al is not convincing because the erythrocytes shown are likely to be extravascular. Extravasated erythrocytes and hemorrhage are such common features of tumors, including uveal melanomas, that the location of erythrocytes cannot be assumed to define the interior of blood vessels.[26–31] Hemorrhage in uveal melanomas can occur during surgical removal of the eye as well as spontaneously.[26,31] Recent studies have begun to elucidate the mechanism of the propensity for hemorrhage in tumors.[32] Therefore, the presence of erythrocytes next to tumor cells does not justify an inference of tumor cell-lined vascular channels.

Third, because cellular membranes are not preserved in the electron micrographs shown in Maniotis et al, cellular boundaries cannot be seen, and it is unclear how many and what types of cells are present in addition to the tumor cells.

Tumor Cells Next to the Vessel Lumen Must Contact Endothelial Cells Somewhere

If blood vessels in uveal melanomas are lined by tumor cells, somewhere there must be a junction between tumor cells in contact with the vessel lumen and endothelial cells. The identification of the junction between the two systems would provide the "smoking gun" in the list of evidence for tumor cell-lined vessels. No such junction was identified, described, or discussed.

Tumor Cell-Lined Vessels in Uveal Melanomas, if Present, Are Likely to Be Infrequent

Maniotis et al do not report the number or proportion of presumptive tumor cell-lined blood vessels in aggressive uveal melanomas. Did these structures constitute 1%, 10%, or 100% of the vessels? Only one example is used to illustrate the electron microscopic observations, and the reader is not told the number of presumptive vessels or the number of tumors that were examined in this way. Because no data were presented that would limit the interpretation to a particular subset of blood vessels, the reader is led to believe that all of the vessels in these tumors are lined by tumor cells. Yet this inference is inconsistent with the results of numerous earlier studies, including some from the same group, that have identified endothelial cells in vessels of aggressive uveal melanomas.[4,6–10,16,18] As mentioned above, three different methods (lectin staining, immunohistochemistry, and transmission electron microscopy) have given consistent and complementary results: most blood vessels in aggressive uveal melanomas are lined by endothelial cells and have the same general features as vessels in other tumors. If tumor cell-lined vessels are present in these tumors, they must be infrequent.

How Novel?

The possibility that cancer cells participate in the formation of blood vessels in tumors has been recognized for many years. It is not surprising that cancer cells with features of endothelial cells line blood vessels of tumors of vascular origin such as angiosarcomas.[33] However, cancer cells have been reported to line vessels in other types of tumors as well. In his book on the pathology of tumors published in 1948, Willis states that "in rapidly growing tumors, [vessels] consist of little more than irregular channels lined by endothelium only or by naked tumor cells."[34] Although Willis does not use the terminology of vasculogenic mimicry, he clearly sets out the concept that tumor cells can acquire a new phenotype and participate in the formation of blood vessels. In the 1960s and 1970s, François,[35,36] Jensen,[37,38] and Duke-Elder and Perkins[26] reported that tumor cells in some uveal melanomas line cavernous spaces or cyst-like blood lakes that may communicate with the microvasculature. Warren,[39] Prause and Jensen,[40] and Hammersen[41] subsequently added ultrastructural evidence of the contribution of cancer cells to the walls of tumor vessels. Warren[39] included "blood vessels without endothelial lining" among his nine categories of tumor vessels. In 1989, Konerding et al[42] used the term endothelial imitation to describe the role of tumor cells in the formation of vascular channels. This concept has been addressed in reviews on tumor blood flow[43,44] and in a textbook of general pathology.[45] Another example of cells other than endothelial cells that form blood vessels can be found in placental cytotrophoblasts creating hybrid fetal/material vessels of the endometrium through a process referred to as pseudo-vasculogenesis.[46] Neither the paper by Maniotis et al nor the two commentaries acknowledges any of these precedents.

How Significant?

The contribution of cancer cells to the formation and lining of blood vessels in tumors has broad biological and medical significance, with pathophysiological and therapeutic implications ranging from predisposition to blood-borne spread of tumor cells, to facilitated entry of drugs into tumors, to the efficacy of conventional anti-cancer drugs as anti-angiogenic agents, and to potential ineffectiveness of endothelial cell-targeted angiogenesis inhibitors.

Because of the potential significance, the issue of endothelial cells *versus* cancer cells lining blood vessels of tumors needs careful, systematic investigation. Earlier studies have all faced the problems of distinguishing between tumor cells and endothelial cells and between intravascular and extravascular erythrocytes. The issue has only begun to be studied with the broad range of contemporary methods such as reporter genes that uniquely identify tumor cells and endothelial cells, and markers that unambiguously label the vessels through which blood circulates. There is also a need to determine the magnitude of the contribution of tumor cells to blood

vessels and of such vessels to tumor blood flow, using quantitative methods with appropriate sampling. By growing human uveal melanomas in immunodeficient mice, it would be possible to label the tumor cells and vasculature with distinctive markers, visualize them by intravital microscopy, and preserve the tissues under optimal conditions for morphological and morphometric examination.

Even though evidence that cancer cells can become lining cells and participate in the formation of blood vessels in tumors has been discussed for many years, the extent and pathophysiological significance of this phenomenon are still unclear and can only be determined by rigorous examination of the issue. Compelling evidence that supports or refutes the concept would be timely and welcome. Unfortunately, the paper by Maniotis et al promised a new level of understanding but did not solve this vexing problem.

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